β1,3-GALACTOSYLTRANSFERASE AND DNA ENCODING THE SAME

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a protein having a β1,3-galactosyltransferase activity, a DNA encoding the protein, a recombinant DNA containing the DNA, a transformant containing the recombinant DNA, a method for producing a protein having a β1,3-galactosyltransferase activity using the transformant, and a method for producing a galactose-containing carbohydrate using the transformant.

2. Background Art

Regarding β1,3-galactosyltransferase genes, the genes derived from higher animal (*J. Biol. Chem.*, *273*: 58 (1998), *J. Biol. Chem.*, *273*: 12770 (1998), *J. Biol. Chem.*, *274*: 12499 (1999)) have been obtained. However, since it is generally difficult to express the genes derived from higher-animal as active proteins in microorganisms, a β1,3-galactosyltransferase gene derived from higher-animal has not been expressed as an active protein in a microorganism such as *Escherichia coli* or the like.

On the other hand, in microorganisms, there is a report stating that a β1,3-galactosyltransferase gene was obtained from *Campylobacter jejuni* and the gene was expressed in *Escherichia coli*. However, although this

enzyme has an activity of transferring galactose to *N*-acetylgalactosamine, there is no report about the activity of transferring galactose to *N*-acetylglucosamine (*J. Biol. Chem.*, *275*: 3896 (2000)).

Human milk abundantly contains galactose-containing carbohydrates, lacto-*N*-tetraose being one of the main components (*Acta Paediatr.*, *82*: 903 (1993), *J. Pediatr. Gastroenterol. Nutr.*, *30*: 181 (2000)). Since it is known that lacto-*N*-tetraose and lacto-*N*-neotetraose, which are the galactose-containing carbohydrates, are recognized by *Pseudomonas aeruginosa* (*Infect. Immun.*, *59*: 700 (1991)), the galactose-containing carbohydrates are considered to be strong candidates for safe antiinfection drugs which can prevent human body from infection with *Pseudomonas aeruginosa*.

Regarding production of a galactose-containing carbohydrate such as lacto-*N*-tetraose or the like, both the methods of extraction from human milk and chemical synthesis are known but such methods have problems in terms of cost and productivity, so that its industrial production method has not yet been established.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a protein having a β1,3-galactosyltransferase activity, and a DNA encoding the protein.

Another object of the present invention is to provide a method for producing a protein having a β1,3-galactosyltransferase activity using a transformant containing the DNA, and a method for producing a galactose-containing carbohydrate using the protein.

These and other objects have been attained by the present invention which relates to a *Streptococcus agalactiae* protein having a β1,3-galactosyltransferase activity, a DNA encoding the protein, a recombinant DNA comprising the DNA, a transformant containing the recombinant DNA, a method for producing a protein having a β1,3-galactosyltransferase activity using the transformant, and a method for producing a galactose-containing carbohydrate using the transformant.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 shows the structure of capsular polysaccharide biosynthesis genes in *Streptococcus agalactiae* Type Ia and Type Ib.

Fig. 2 shows the construction steps of β1,3-galactosyltransferase plasmids pBBPIJ and pBBPJ.

DETAILED DESCRIPTION OF THE INVENTION

This application is based on Japanese application No. 2001-392, filed on January 5, 2001, the entire content of which is incorporated herein by reference.

In order to solve the above problems, the present inventors have conducted intensive studies and found a novel β1,3-galactosyltransferase, among enzymes concerning capsular polysaccharide biosynthesis in *Streptococcus agalactiae*, and have isolated the DNA encoding such enzyme.

Specifically, the present invention relates to the following (1) to (19):

(1) A protein having a β1,3-galactosyltransferase activity derived from a microorganism having an activity of transferring galactose to *N*-acetylglucosamine with β1,3-linkage.

(2) The protein according to (1), wherein the microorganism belongs to the genus *Streptococcus*.

(3) The protein according to (2), wherein the microorganism is *Streptococcus agalactiae*.

(4) A protein comprising the amino acid sequence represented by SEQ ID NO:1.

(5) A protein comprising an amino acid sequence in which at most 20 amino acids are deleted, replaced, inserted or added in the amino acid sequence represented by SEQ ID NO:1, said protein having a β1,3-galactosyltransferase activity.

(6) A DNA encoding the protein of any one of (1) to (5).

(7) A DNA comprising the nucleotide sequence represented by SEQ ID NO:2.

(8) A DNA which hybridizes with a DNA comprising the complementary sequence to the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions, and encodes a protein having a β1,3-galactosyltransferase activity.

(9) A recombinant DNA comprising the DNA of any one of (6) to (8) and a vector.

(10) A transformant obtained by introducing the recombinant DNA of (9) into a host cell.

(11) The transformant according to (10), wherein the host cell is a microorganism.

(12) The transformant according to (11), wherein the microorganism belongs to the genus *Escherichia*.

(13) The transformant according to (12), wherein the microorganism belonging to the genus *Escherichia* is *Escherichia coli*.

(14) A method for producing a protein having a β1,3-galactosyltransferase activity, comprising:

culturing the transformant of any one of (10) to (13) in a medium to produce and accumulate a protein having a β1,3-galactosyltransferase activity in the culture, and

recovering the protein from the culture.

(15) A method for producing a galactose-containing carbohydrate, comprising:

selecting, as an enzyme source, a culture of the transformant of any one of (10) to (13) or a treated product of the culture,

allowing the enzyme source, uridine-5'-diphosphogalactose and an acceptor carbohydrate to be present in an aqueous medium to produce and accumulate the galactose-containing carbohydrate in the aqueous medium, and

recovering the galactose-containing carbohydrate from the aqueous medium.

(16) The method according to (15), wherein the treated product of the culture is selected from the group consisting of a concentrated product of the culture, a dried product of the culture, cells obtained by centrifuging the culture, a dried product of the cells, a freeze-dried product of the cells, a surfactant-treated product of the cells, an ultrasonic-treated product of the cells, a mechanically disrupted product of the cells, a solvent-treated product of the cells, an enzyme-treated product of the cells, a protein fraction of the cells, an immobilized product of the cells and an enzyme preparation obtained by extracting from the cells.

(17) The method according to (15), wherein the acceptor carbohydrate is a carbohydrate having *N*-acetylglucosamine at its non-reducing terminal.

(18) The method according to (15), wherein the acceptor carbohydrate is selected from the group consisting of *N*-acetylglucosamine and lacto-*N*-triose II.

(19) The method according to (15), wherein the galactose-containing carbohydrate is selected from the group consisting of lacto-*N*-biose and lacto-*N*-tetraose.

The protein having a β1,3-galactosyltransferase activity of the present invention is a protein having a β1,3-galactosyltransferase activity derived from a microorganism which uses, as a substrate, an acceptor carbohydrate having *N*-acetylglucosamine (hereinafter referred to as "GlcNAc") on its non-reducing terminal. For example, preferred is a protein having a β1,3-galactosyltransferase activity derived from a microorganism belonging to the genus *Streptococcus*, and more preferred is a protein having a β1,3-galactosyltransferase activity derived from *Streptococcus agalactiae*.

Specifically, the protein of the present invention includes a protein comprising the amino acid sequence represented by SEQ ID NO:1, and a protein comprising an amino acid sequence in which at most 20 amino acids are deleted, replaced, inserted or added in the amino acid sequence represented by SEQ ID NO:1 and having a β1,3-galactosyltransferase activity.

The modified protein can readily be obtained using a method for introducing site-directed mutation(s) described in, for example, *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as "*Molecular Cloning*, 2nd ed."), *Current Protocols in Molecular Biology*, John Wiley & Sons (1987-1997) (hereinafter referred to as "*Current Protocols in Molecular Biology*"), *Nuc. Acids. Res.*, *10*: 6487 (1982), *Proc. Natl. Acad. Sci. USA*, *79*: 6409 (1982), *Gene*, *34*: 315 (1985), *Nuc. Acids. Res.*, *13*: 4431 (1985), *Proc. Natl. Acad. Sci. USA*, *82*: 488 (1985) and the like. For example, the protein can be obtained by introducing mutation(s) to DNA encoding a protein comprising the amino acid sequence represented by SEQ ID NO:2.

The number of the amino acids which are deleted, replaced, inserted or added is not particularly limited; however, it is usually 1 to 20, preferably 1 to 10, and more preferably 1 to 5, amino acids.

The at most 20 amino acid deletion, replacement, insertion or addition in the amino acid sequence of the protein of the present invention is used herein to refer to that at most 20 amino acids are deleted, replaced, inserted or added to at one or plural positions in the amino acid sequence. The deletion, replacement, insertion or addition may be caused in the same amino acid sequence simultaneously. Also, the amino acid residue replaced,

inserted or added can be natural or non-natural. Examples of the natural amino acid residue include L-alanine, L-asparagine, L-asparatic acid, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-cysteine, and the like.

Herein, examples of amino acid residues which are replaced with each other are shown below. Amino acid residues in the same group can readily be replaced with each other.

Group A:

leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, O-methylserine, t-butylglycine, t-butylalanine, cyclohexylalanine;

Group B:

asparatic acid, glutamic acid, isoasparatic acid, isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid;

Group C:

asparagine, glutamine;

Group D:

lysine, arginine, ornithine, 2,4-diaminobutanoic acid, 2,3-diaminopropionic acid;

Group E:

proline, 3-hydroxyproline, 4-hydroxyproline;

Group F:

serine, threonine, homoserine;

Group G:

phenylalanine, tyrosine.

Also, in order to have the β1,3-galactosyltransferase activity of the protein of the present invention, it has preferably at least 50% or more, preferably 60% or more, still more preferably 80% or more, most preferably 95% or more, of identity to the amino acid sequence represented by SEQ ID NO:1. The identity of a nucleotide sequence or an amino acid sequence can be determined using the algorithm "BLAST" by Karlin and Altschl (*Proc. Natl. Acad. Sci. USA*, *90*: 5873-5877 (1993)). The programs called "BLASTN" and "LASTX" have developed based on the above algorithm (*J. Mol. Biol.*, *215*: 403-410 (1990)). In the case of analyzing a nucleotide sequence based on BLAST, the parameter can be set to e.g. score = 100, wordlength = 12. And in the case of analyzing an amino acid sequence based on BLASTX, the parameter can be set to e.g. score = 50, wordlength = 3. In the case of using BLAST or Gapped BLAST program, a default parameter of each program can be used. The specific analysis methods of using the above programs are known in the art (http://www.ncbi.nlm.nih.gov.).

The DNA of the present invention includes a DNA encoding the protein of the present invention.

Specific examples include a DNA encoding a protein comprising the amino acid sequence represented by SEQ ID NO:1, a DNA comprising the nucleotide sequence represented by SEQ ID NO:2, and a DNA which hybridizes with a DNA comprising the complementary sequence to the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions and encodes a protein having a β1,3-galactosyltransferase activity.

The DNA which hybridizes under stringent conditions is a DNA obtained by colony hybridization, plaque hybridization, Southern hybridization or the like using, as a probe, the DNA comprising the complementary sequence to the nucleotide sequence represented by SEQ ID NO:2. Specific examples include a DNA which can be identified by carrying out hybridization at 65°C in the presence of 0.7-1.0 M NaCl using a filter on which a DNA prepared from colonies or plaques is immobilized, and then washing the filter with 0.1× to 2× SSC solution (the composition of 1× SSC contains 150 mM sodium chloride and 15 mM sodium citrate) at 65°C.

The hybridization can be carried out in accordance with a known method described in, for example, *Molecular Cloning*, 2nd ed., *Current Protocols in Molecular Biology*, *DNA Cloning 1: Core Techniques, A Practical Approach*, 2nd ed., Oxford University (1995) or the like. Specific examples of the DNA which can be hybridized include a DNA

having an identity of 60% or more, preferably 80% or more, and more preferably 95% or more, with the complementary sequence to the nucleotide sequence represented by SEQ ID NO:2 when calculated using above BLAST or the like.

The transformant which produces the protein of the present invention having a β1,3-galactosyltransferase activity can be obtained, e.g., by preparing a recombinant DNA through ligation of the DNA of the present invention to a vector DNA in accordance with the method described in *Molecular Cloning*, 2nd ed., and then transforming a host cell with the recombinant DNA in accordance with the method described in *Molecular Cloning*, 2nd ed.

The present invention is explained below in more detail.

(1) Preparation of the DNA of the present invention

The DNA of the present invention is desirably prepared from a microorganism belonging to the genus *Streptococcus*. Examples of the microorganism belonging to the genus *Streptococcus* include *Streptococcus agalactiae*, such as *Streptococcus agalactiae* Type Ib and the like.

The microorganism belonging to the genus *Streptococcus* is cultured by a known method (for example, the method described in *J. Bacteriol.*, *181*: 5176 (1999)).

After culturing, chromosomal DNA of the microorganism is isolated and purified by a known method

(for example, method described in *Current Protocols in Molecular Biology*).

A fragment containing the DNA of the present invention can be obtained by a hybridization method, PCR or the like using a synthetic DNA designed based on a nucleotide sequence among the capsular polysaccharide biosynthesis genes of *Streptococcus agalactiae* Type III or Type Ia.

The vector to which the DNA is ligated may be any vector, such as a phage vector, a plasmid vector or the like, so long as it can replicate autonomously in *Escherichia coli* K12. Specific examples include ZAP Express (manufactured by Stratagene, *Strategies*, *5*: 58 (1992)), pBluescript II SK(+) (manufactured by Stratagene, *Nucleic Acids Res.*, *17*: 9494 (1989)), λzap II (manufactured by Stratagene), λgt10 and λgt11 (*DNA Cloning, A Practical Approach*, *1*: 49 (1985)), λTriplEx (manufactured by Clontech), λExCell (manufactured by Amersham Pharmacia Biotech), pUC18 (*Gene*, *33*: 103 (1985)) and the like.

Any microorganism belonging to *Escherichia coli* can be used for the host of the recombinant DNA obtained by ligating the DNA of the present invention to the above vector, so long as it is a microorganism belonging to *Escherichia coli*. Specific examples include *Escherichia coli* XL1-Blue MRF' (manufactured by Stratagene, *Strategies*, *5*: 81 (1992)), *Escherichia coli* C600 (*Genetics*, *39*: 440

(1954)), *Escherichia coli* Y1088 (*Science*, *222*: 778 (1983)), *Escherichia coli* Y1090 (*Science*, *222*: 778 (1983)), *Escherichia coli* NM522 (*J. Mol. Biol.*, *166*: 1 (1983)), *Escherichia coli* K802 (*J. Mol. Biol.*, *16*: 118 (1966)), *Escherichia coli* JM105 (*Gene*, *38*: 275 (1985)) and the like.

Any method can be used in the method for introducing the recombinant DNA, so long as it is a method for introducing DNA into the selected host cell. Examples include a method using a calcium ion (*Proc. Natl. Acad. Sci. USA*, *69*: 2110 (1972)), a protoplast (Japanese Published Unexamined Patent Application No. 248394/88), an electroporation (*Nucleic Acid Res.*, *16*: 6127 (1988)) and the like.

The nucleotide sequence of the DNA of the present invention contained in the recombinant DNA can be determined by extracting the recombinant DNA from the thus obtained transformant. For the determination of the nucleotide sequence, a conventional method, such as the dideoxy method (*Proc. Natl. Acad. Sci. USA*, *74*: 5463 (1977)) or an apparatus for nucleotide sequence analysis, such as DNA Sequencer 373A (manufactured by Perkin-Elmer) or the like, can be used.

The DNA of interest can also be prepared by chemical synthesis based on the thus determined nucleotide sequence using, for example, DNA Synthesizer 8905 Type manufactured by Perceptive Biosystems or the like.

Examples of transformant containing the thus obtained recombinant DNA include *Escherichia coli* JM109/pBBPJ containing a plasmid DNA having the nucleotide sequence represented by SEQ ID NO:2.

(2) Preparation of the protein of the present invention.

The protein of the present invention can be produced by expressing the DNA of the present invention obtained by the method of (1) in a host cell, for example, as shown below, using a method described in *Molecular Cloning*, 2nd ed., *Current Protocols in Molecular Biology* or the like.

When the DNA of the present invention is used, a DNA fragment of a suitable length containing a portion which encodes the protein of the present invention can be prepared, if necessary. In addition, productivity of the protein can be improved by substituting a nucleotide of the protein-coding portion of the nucleotide sequence so that it has the most suitable codons for the expression in the host.

The transformant which expresses the DNA of the present invention can be obtained by inserting the DNA into a downstream of the promoter of a suitable expression vector to thereby prepare a recombinant DNA, and introducing the recombinant DNA into a host cell suitable for the expression vector.

Any bacteria, yeasts, animal cells, insect cells, plant cells, and the like can be used as the host cell so long as it can express the gene of interest.

Examples of the expression vector include those which can replicate autonomously in the above-described host cell or can be integrated into chromosome and have a promoter at such a position that the DNA of the present invention can be transcribed.

When a procaryote cell, such as a bacterium or the like, is used as the host cell, it is preferred that the recombinant DNA containing the DNA of the present invention can replicate autonomously in the bacterium. It is also preferred that the recombinant vector contains a promoter, a ribosome binding sequence, the DNA of the present invention and a transcription termination sequence. A gene regulating the promoter may also be desirably contained therewith in operable combination.

Examples of the expression vector include pHelix1 (manufactured by Roche Diagnostics), pKK223-3 (manufactured by Amersham Pharmacia Biotech), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pET-3 (manufactured by Novagen), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 (*Agric. Biol. Chem.*, *48*: 669 (1984)), pLSA1 (*Agric. Biol. Chem.*, *53*: 277 (1989)), pGEL1 (*Proc. Natl. Acad. Sci. USA*, *82*: 4306 (1985)), pBluescript II

SK(+) (manufactured by Stratagene), pTrs30 (prepared from *Escherichia coli* JM109/pTrS30 (FERM BP-5407)), pTrs32 (prepared from *Escherichia coli* JM109/pTrS32 (FERM BP-5408)), pPAC31 (WO 98/12343), pUC19 (*Gene*, *33*: 103 (1985)), pSTV28 (manufactured by Takara Shuzo), pUC118 (manufactured by Takara Shuzo), pPA1 (Japanese Published Unexamined Patent Application No. 233798/88), and the like.

Any promoter can be used so long as it can function in the host cell. Examples include promoters derived from *Escherichia coli*, phage and the like, such as *trp* promoter (P*trp*), *lac* promoter (P*lac*), P_L promoter, P_R promoter, P_{SE} promoter, *etc.*, SPO1 promoter, SPO2 promoter, penP promoter and the like. Also, artificially designed and modified promoters, such as a promoter in which two P*trp* are linked in tandem (P*trp*×2), *tac* promoter, *lac*T7 promoter *let*I promoter and the like, can be used.

It is preferred to use a plasmid in which the space between Shine-Dalgarno sequence, which is the ribosome binding sequence, and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 base pairs).

The transcription termination sequence is not always necessary for the expression of the DNA of the present invention. However, it is preferred to provide a transcription terminating sequence just downstream of the structural gene.

Examples of the procaryote cell include microorganisms belonging to the genera *Escherichia*, *Serratia*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Microbacterium*, *Pseudomonas*, *Streptococcus* and the like. Specific examples include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Corynebacterium ammoniagenes*, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 14067, *Corynebacterium glutamicum* ATCC 13869, *Corynebacterium acetoacidophilum* ATCC 13870, *Microbacterium ammoniaphilum* ATCC 15354, *Pseudomonas* sp. D-0110, *Streptococcus agalactiae* Type Ia, *Streptococcus agalactiae* Type Ib, *Streptococcus agalactiae* Type III, *Streptococcus pneumoniae* Type 14, and the like.

With regard to the method for the introduction of the recombinant DNA, any method for introducing DNA into the above-described host cells, such as a method using a calcium ion (*Proc. Natl. Acad. Sci. USA*, *69*: 2110 (1972)), a protoplast (Japanese Published Unexamined Patent

Application No. 248394/88), an electroporation (*Nucleic Acids Res.*, *16*: 6127 (1988)) and the like, can be used.

When yeast is used as the host cell, examples of the expression vector include YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), pHS19, pHS15, and the like.

Any promoter can be used so long as it can function in yeast. Examples include PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, a heat shock polypeptide promoter, MFα1 promoter, CUP 1 promoter and the like.

Examples of the host cell include yeast strains belonging to the genera *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, *Trichosporon*, *Schwanniomyces*, *Pichia*, *Candida* and the like. Specific examples include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans*, *Schwanniomyces alluvius*, *Pichia pastoris*, *Candida utilis* and the like.

With regard to the method for the introduction of the recombinant DNA, any method for introducing DNA into yeast, such as an electroporation (*Methods. Enzymol.*, *194*: 182 (1990)), a spheroplast method (*Proc. Natl. Acad. Sci. USA*, *75*: 1929 (1978)), a lithium acetate method (*J. Bacteriol.*, *153*: 163 (1983)) and the like, can be used.

When an animal cell is used as the host cell, examples of the expression vector include pcDNAI and pcDM8 (manufactured by Funakoshi), pAGE107 (Japanese Published Unexamined Patent Application No. 22979/91), pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90), pcDM8 (*Nature*, *329*: 840 (1987)), pcDNAI/Amp (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 (*J. Biochem.*, *101*: 1307 (1987)), pAGE210, pAMo, pAMoA and the like.

Any promoter can be used so long as it can function in an animal cell. Examples include a promoter of IE (immediate early) gene of cytomegalovirus (CMV), an early promoter of SV40, a metallothionein promoter, a promoter of retrovirus, a heat shock promoter, SRα promoter, and the like. Also, the enhancer of the IE gene of human CMV can be used together with the promoter.

Examples of the host cell include mouse myeloma cell, rat myeloma cell, mouse hybridoma cell, human Namalwa cell, Namalwa KJM-1 cell, human fetal kidney cell, human leukemia cell, African grivet kidney cell, Chinese hamster ovary (CHO) cell HST5637 (Japanese Published Unexamined Patent Application No. 299/88) and the like.

Examples of the mouse myeloma cell include SP2/0, NS0 and the like. Examples of the rat myeloma cell include YB2/0 and the like. Examples of the human fetal kidney cell include HEK293 (ATCC: CRL-1573) and the like.

Examples of the human leukemia cell include BALL-1 and the like. Examples of the African grivet kidney cell include COS-1, COS-7 and the like.

The method for introduction of the recombinant DNA into animal cells is not particularly limited, so long as it is the general method for introducing DNA into animal cells, such as an electroporation (*Cytotechnology*, *3*: 133 (1990)), a calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), a lipofection (*Proc. Natl. Acad. Sci. USA*, *84*, 7413 (1987)), the method described in *Virology*, *52*: 456 (1973) and the like.

When an insect cell is used as the host cell, the protein can be expressed by a known method described in, for example, *Baculovirus Expression Vectors, A Laboratory Manual,* W.H. Freeman and Company, New York (1992), *Molecular Biology, A Laboratory Manual*, *Current Protocols in Molecular Biology*, *Bio/Technology*, *6*: 47 (1988) or the like.

Specifically, a transfer vector containing the DNA to make it express and baculovirus are co-transfected into an insect cell to obtain a recombinant virus in a supernatant of the culture of its insect cell, and then an insect cell is infected with the resulting recombinant virus to express the protein.

Examples of the transfer vector used in the method include pVL1392, pVL1393 and pBlueBacIII (all manufactured by Invitrogen), and the like.

Examples of the baculovirus include *Autographa californica* nuclear polyhedrosis virus which infects insects of the family *Barathra* and the like.

Examples of the insect cell include *Spodoptera frugiperda* ovary cell, *Trichoplusia ni* ovary cell, *Bombyx mori* ovary-derived culturing cell and the like.

Examples of *Spodoptera frugiperda* ovary cells include Sf9 and Sf21 (*Baculovirus Expression Vectors, A Laboratory Manual*) and the like. Examples of *Trichoplusia ni* ovary cells include High 5 and BTI-TN-5B1-4 (manufactured by Invitrogen) and the like. Examples of the cell line derived from silkworm ovary cellinclude *Bombyx mori* N4 and the like.

The methods for co-transfecting the above transfer vector and the above baculovirus for the preparation of the recombinant virus include a calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), a lipofection (*Proc. Natl. Acad. Sci. USA*, *84*: 7413 (1987)), and the like.

When a plant cell is used as the host cell, examples of expression vector include Ti plasmid, a tobacco mosaic virus vector, and the like.

Any promoter can be used so long as it can function in a plant cell. Examples include 35S promoter of cauliflower mosaic virus (CaMV), rice actin 1 promoter, and the like.

Examples of the host cells include plant cells and the like, such as tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, barley, and the like.

The method for introducing the recombinant DNA is not particularly limited, so long as it is the general method for introducing DNA into a plant cell, such as the *Agrobacterium* method (Japanese Published Unexamined Patent Application No. 140885/84, Japanese Published Unexamined Patent Application No. 70080/85, WO 94/00977), the electroporation (Japanese Published Unexamined Patent Application No. 251887/85), the particle gun method (Japanese Patents 2606856 and 2517813), and the like.

The gene can be expressed as a secretory protein or a fusion protein and the like in accordance with the methods described in *Molecular Cloning*, 2nd ed., in addition to direct expression.

When expressed in yeast, an animal cell or an insect cell, a glycosylated protein can be obtained.

The protein of the present invention can be produced by culturing the thus obtained transformant of the present invention in a medium to produce and accumulate the

protein in the culture, and recovering the protein from the culture.

Culturing of the transformant of the present invention in a medium is carried out according to the conventional method as used in culturing of the host.

As a medium for culturing the transformant obtained by using, as the host, prokaryote (such as *Escherichia coli* or the like) or eukaryote (such as yeast or the like), the medium may be either a natural medium or a synthetic medium, so long as it contains a carbon source, a nitrogen source, an inorganic salt and the like which can be assimilated by the organism and the transformant can be cultured efficiently.

Examples of the carbon source which can be assimilated by the transformant include carbohydrates (for example, glucose, fructose, sucrose, molasses containing them, starch, starch hydrolysate, *etc.*), organic acids (for example, acetic acid, propionic acid, *etc.*), alcohols (for example, ethanol, propanol, *etc.*), and the like.

Examples of the nitrogen source include ammonia, various ammonium salts of inorganic acids or organic acids (for example, ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, *etc.*), other nitrogen-containing compounds, peptone, meat extract, yeast extract, corn steep liquor, casein hydrolysate, soybean meal and

soybean meal hydrolysate, various fermented cells and hydrolysates thereof, and the like.

Examples of the inorganic salt include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, and the like.

Culturing is usually carried out under aerobic conditions by shaking culture, submerged spinner culture under aeration or the like. The culturing temperature is preferably from 15 to 40°C, and the culturing time is generally from 5 hours to 7 days. The pH of the medium is preferably maintained at 3.0 to 9.0 during culturing. The pH can be adjusted using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia, or the like.

Also, antibiotics such as ampicillin, tetracycline, and the like, can be added to the medium during culturing, if necessary.

When a microorganism transformed with an expression vector containing an inducible promoter is cultured, an inducer can be added to the medium, if necessary. For example, isopropyl-β-D-thiogalactopyranoside (IPTG) or the like can be added to the medium when a microorganism transformed with an expression vector containing *lac* promoter is cultured, or indoleacrylic acid or the like can

by added thereto when a microorganism transformed with an expression vector containing *trp* promoter is cultured.

Examples of the medium for culturing a transformant obtained using an animal cell as the host include generally used RPMI 1640 medium (*The Journal of the American Medical Association, 199*: 519 (1967)), Eagle's MEM (*Science, 122*: 501 (1952)), DMEME (*Virology, 8*: 396 (1959)), and 199 Medium (*Proceeding of the Society for the Biological Medicine, 73*: 1 (1950)), as well as other media to which fetal calf serum or the like has been added to the above media and the like.

Culturing is generally carried out under conditions at pH 6 to 8 and at 30 to 40°C for 1 to 7 days in the presence of 5% CO_2 or the like.

Furthermore, if desired, antibiotics such as kanamycin, penicillin, streptomycin and the like, can be added to the medium during culturing.

Examples of the medium for culturing a transformant obtained using an insect cell as the host include generally used TNM-FH medium (manufactured by Pharmingen), Sf-900 II SFM (manufactured by Life Technologies), ExCell 400 and ExCell 405 (both manufactured by JRH Biosciences), Grace's Insect Medium (*Nature, 195*: 788 (1962)) and the like.

Culturing is generally carried out under conditions at pH 6 to 7 and at 25 to 30°C for 1 to 5 days or the like.

Furthermore, if desired, antibiotics such as gentamicin and the like, can be added to the medium during culturing.

A transformant obtained using a plant cell as the host cell can be used as the cell or after differentiating to a plant cell or organ. Examples of the medium used in culturing of the transformant include Murashige and Skoog (MS) medium, White medium, media to which a plant hormone, such as auxin, cytokinine, or the like has been added, and the like.

Culturing is carried out generally at a pH 5 to 9 and at 20 to 40°C for 3 to 60 days.

Also, antibiotics such as kanamycin, hygromycin and the like, can be added to the medium during culturing, if necessary.

As described above, the protein can be produced by culturing a transformant derived from a microorganism, animal cell or plant cell containing a recombinant DNA to which a DNA encoding the protein of the present invention has been inserted according to the general culturing method to produce and accumulate the protein, and recovering the protein from the culture.

The method for producing the protein of the present invention includes a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, or a method of production on an outer membrane

of the host cell. The method can be selected by changing the host cell employed or the structure of the protein produced.

When the protein of the present invention is produced in a host cell or on an outer membrane of the host cell, the protein can be actively secreted extracellularly according to, for example, the method of Paulson *et al.* (*J. Biol. Chem.*, *264*: 17619 (1989)), the method of Lowe *et al.* (*Proc. Natl. Acad. Sci. USA*, *86*: 8227 (1989); *Genes Develop.*, *4*: 1288 (1990)), or the methods described in Japanese Published Unexamined Patent Application Nos. 336963/93, 823021/94, and the like.

Specifically, the protein of the present invention can be actively secreted extracellularly by expressing it in the form that a signal peptide has been added to the side of N-terminal of a protein containing an active site of the protein of the present invention according to the recombinant DNA technique.

Furthermore, the amount produced can be increased using a gene amplification system, such as by use of a dihydrofolate reductase gene or the like according to the method described in Japanese Published Unexamined Patent Application No. 227075/90.

Moreover, the protein of the present invention can be produced by a transgenic animal (transgenic nonhuman animal) or plant (transgenic plant).

When the transformant is the nonhuman animal individual or plant individual, the protein of the present invention can be produced by breeding or cultivating it so as to produce and accumulate the protein, and recovering the protein from the nonhuman animal individual or plant individual.

Examples of the method for producing the protein of the present invention using the nonhuman animal individual include a method for producing the protein of the present invention in a nonhuman animal developed by introducing a gene according to known methods (*Am. J. Clin. Nutr.*, *63*: 639S (1996), *Am. J. Clin. Nutr.*, *63*: 627S (1996), *Bio/Technology*, *9*: 830 (1991)).

In the nonhuman animal individual, the protein can be produced by breeding a transgenic nonhuman animal to which the DNA encoding the protein of the present invention has been introduced to produce and accumulate the protein in the animal, and recovering the protein from the animal. Examples of the production and accumulation place in the animal include milk (Japanese Published Unexamined Patent Application No. 309192/88), egg and the like of the animal. Any promoter can be used, so long as it can function in the animal. Suitable examples include an α-casein promoter, a β-casein promoter, a β-lactoglobulin promoter, a whey acidic protein promoter, and the like, which are specific for mammary glandular cells.

Examples of the method for producing the protein of the present invention using the plant individual include a method for producing the protein of the present invention by cultivating a transgenic plant to which the DNA encoding the protein of the present invention is introduced by a known method (*Tissue Culture*, *20* (1994), *Tissue Culture*, *21* (1994), *Trends in Biotechnol.*, *15*: 45 (1997)) to produce and accumulate the protein in the plant, and recovering the protein from the plant.

The protein produced by the transformant of the present invention can be isolated and purified using the general method for isolating and purifying an enzyme.

For example, when the protein of the present invention is expressed as a soluble product in the host cells, the cells are collected by centrifugation after culturing, suspended in an aqueous buffer, and disrupted using an ultrasonicator, a French press, a Manton Gaulin homogenizer, a Dynomill, or the like to obtain a cell-free extract.

From the supernatant obtained by centrifuging the cell-free extract, a purified product can be obtained by the general method used for isolating and purifying an enzyme, for example, solvent extraction, salting-out using ammonium sulfate or the like, desalting, precipitation using an organic solvent, anion exchange chromatography using a resin, such as diethylaminoethyl (DEAE)-Sepharose,

DIAION HPA-75 (manufactured by Mitsubishi Chemical) or the like, cation exchange chromatography using a resin, such as S-Sepharose FF (manufactured by Pharmacia) or the like, hydrophobic chromatography using a resin, such as butyl sepharose, phenyl sepharose or the like, gel filtration using a molecular sieve, affinity chromatography, chromatofocusing, or electrophoresis, such as isoelectronic focusing or the like, alone or in combination thereof.

When the protein is expressed as an inclusion body in the host cells, the cells are collected in the same manner, disrupted and centrifuged to recover the protein as the precipitate fraction. Next, the inclusion body of the protein is solubilized with a protein-denaturing agent.

The solubilized protein solution is diluted or dialyzed to lower the concentration of the protein denaturing agent in the solution. Thus, the normal tertiary structure of the protein is reconstituted. After the procedure, a purified product of the protein can be obtained by a purification/isolation method similar to the above.

When the protein of the present invention or its glycosylated-derivative is secreted out of cells, the protein or its derivative can be collected in the culture supernatant.

Namely, the culture supernatant is obtained by treating the culture medium in a treatment similar to the

above, such as centrifugation or the like. Then a purified product can be obtained from the supernatant using a purification/isolation method similar to the above.

Examples of the protein obtained by the above method include a protein comprising the amino acid sequence represented by SEQ ID NO:1.

Furthermore, a fusion protein of the protein of the present invention and other protein is produced, and can be purified using affinity chromatography using a substance having affinity to the fusion protein. For example, the protein of the present invention is produced as a fusion protein with protein A according to the method of Lowe *et al.* (*Proc. Natl. Acad. Sci. USA*, *86*: 8227 (1989); *Genes Develop.*, *4*: 1288 (1990)), or the method described in Japanese Published Unexamined Patent Application No. 336963/93 or WO 94/23021, and it can be purified by affinity chromatography using immunoglubulin G.

Moreover, the protein of the present invention is produced as a fusion protein with Flag peptide, and the fusion protein can be purified by affinity chromatography using an anti-Flag antibody (*Proc. Natl. Acad. Sci., USA*, *86*: 8227 (1989)). Further purification can be carried out by affinity chromatography using the antibody against the protein *per se*.

Also, based on the information of the thus obtained protein, the protein of the present invention can be

produced by a chemical synthesis method, such as Fmoc (fluorenylmethyloxycarbonyl) method, tBoc (t-butyloxycarbonyl) method, or the like. It can also be chemically synthesized using a peptide synthesizer manufactured by Advanced ChemTech, Perkin-Elmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, Shimadzu Corporation, or the like.

(3) Preparation of galactose-containing carbohydrate

A galactose-containing carbohydrate can be produced in an aqueous medium using a culture of the transformant obtained by culturing described in (2) or a treated product of the culture as the enzyme source.

Examples of the treated product of culture include a concentrated product of the culture, a dried product of the culture, cells obtained by centrifuging the culture, a dried product of the cells, a freeze-dried product of the cells, a surfactant-treated product of the cells, an ultrasonic-treated product of the cells, a mechanically disrupted product of the cells, a solvent-treated product of the cells, an enzyme-treated product of the cells, a protein fraction of the cells, an immobilized product of the cells, an enzyme preparation obtained by extracting from the cell, and the like.

The enzyme source for use in the production of a galactose-containing carbohydrate is used in a

concentration of 1 mU/l to 1,000 U/l, preferably 10 mU/l to 100 U/l, when the activity capable of forming 1 μmol of galactose-containing carbohydrate at 37°C in 1 minute is defined as 1 unit (U).

Examples of the aqueous medium for use in the production of a galactose-containing carbohydrate include water, buffer solutions (for example, phosphate buffer, carbonate buffer, acetate buffer, borate buffer, citrate buffer, tris bufrer, *etc.*), alcohols (for example, methanol, ethanol, *etc.*), esters (for example, ethyl acetate, *etc.*), ketones (for example, acetone, *etc.*), amides (for example, acetamide, *etc.*), and the like. Also, the culture of the microorganisms used as the enzyme source can be used as an aqueous medium.

In producing a galactose-containing carbohydrate, a surfactant or an organic solvent may be added, if necessary. Any surfactant capable of accelerating the formation of a galactose-containing carbohydrate may be used as the surfactant. Examples include nonionic surfactants (for example, polyoxyethylene octadecylamine (e.g., Nymeen S-215, manufactured by Nippon Oil & Fats), *etc.*), cationic surfactants (for example, cetyltrimethylammonium bromide, alkyldimethyl benzylammoniumchloride (e.g., Cation F2-40E, manufactured by Nippon Oil & Fats), *etc.*), anionic surfactants (for example, lauroyl sarcosinate, *etc.*), tertiary amines (for example, alkyldimethylamine (e.g.,

Tertiary Amine FB, manufactured by Nippon Oil & Fats), *etc.*), and the like, which are used alone or as a mixture of two or more. The surfactant is used generally in a concentration of 0.1 to 50 g/l. Examples of the organic solvent include xylene, toluene, fatty acid alcohol, acetone, ethyl acetate, and the like, which are used in a concentration of generally 0.1 to 50 ml/l.

The galactose-containing carbohydrate formation reaction is carried out in an aqueous medium having a pH 5 to 10, preferably 6 to 8, at 20 to 50°C for 1 to 96 hours. In this formation reaction, inorganic salts, such as $MnCl_2$, $MgCl_2$ and the like, can be added, if necessary.

The amount of the galactose-containing carbohydrate produced in the aqueous medium can be determined, for example, using a High-performance anion-exchange chromatography with pulsed amperometric detection system manufactured by Dionex (*Anal. Biochem.*, *189*: 151 (1990)).

The galactose-containing carbohydrate produced in the aqueous medium can be collected by the ordinary methods using activated carbon, ion exchange resins, and the like.

The present invention is explained based on the examples, but the scope of the present invention is not limited thereto.

Best mode for carrying out the invention

Example 1

Isolation of DNA containing β1,3-galactosyltransferase gene:

(1) Cloning of capsular polysaccharide biosynthesis genes from *Streptococcus agalactiae* Type Ia (1)

Streptococcus agalactiae Type Ia was cultured by the method described in *J. Bacteriol., 181*: 5176 (1999). After collecting the cells by centrifugation, chromosomal DNA of the microorganism was isolated and purified in accordance with the method described in *Current Protocols in Molecular Biology*.

DNAs having the nucleotide sequences represented by SEQ ID NOs:4 and 5 which had been designed based on the nucleotide sequence around the *cps*D gene which is one of the capsular polysaccharide biosynthesis genes of *Streptococcus agalactiae* Type III (*Mol. Microbiol., 8*: 843 (1993)) were synthesized using a DNA synthesizer Model 8905 manufactured by Perceptive Biosystems. Using these synthetic DNAs as a primer set, PCR was carried out using the chromosomal DNA of *Streptococcus agalactiae* Type Ia as the template. The PCR was carried out using 40 μl of a reaction solution containing 0.1 μg of the chromosomal DNA, 0.5 μmol/l each primer, 2.5 units of TaKaRa LA Taq polymerase (manufactured by Takara Shuzo), 4 μl of a buffer solution for TaKaRa LA Taq polymerase and 200 μmol/l each

deoxyNTP and repeating a reaction step consisting of 1 minute at 94°C, 2 minutes at 42°C and 3 minutes at 72°C 30 times. A probe was prepared by labeling the thus amplified fragment using Random Primer DNA Labeling Kit (manufactured by Takara Shuzo).

Fragments obtained by digesting the chromosomal DNA of *Streptococcus agalactiae* Type Ia with a restriction enzyme *Eco*RI were ligated to pBluescript II SK(+) to prepare recombinant DNAs, and a library was prepared by transforming *E. coli* JM109 with these recombinant DNAs.

Using the above probe and library, colony hybridization was carried out according to the method known by persons having ordinary skill in the art to obtain a strain of clone showing a strong signal. A plasmid contained in this strain was named pBA101 and its structure was analyzed to find that it was a structure in which a 3.5 kb fragment derived from the chromosomal DNA of *Streptococcus agalactiae* Type Ia was inserted into pBluescript II SK(+). The nucleotide sequence of the DNA was determined according to the method known by persons having ordinary skill in the art, and as a result, the three genes named *cpsIaF*, *cpsIaG* and *cpsIaH*, and a part of a gene named *cpsIaE* described in *J. Bacteriol.*, *181*: 5176 (1999) were found in the DNA, so that it was confirmed that the DNA contains a part of the capsular polysaccharide biosynthesis genes. Also, as a result of homology search,

it was confirmed that the *cpsIaE* gene and the *cpsIaG* gene had high homology with the glucosyltransferase gene and with the β1,4-galactosyltransferase gene, respectively (Fig. 1).

(2) Cloning of capsular polysaccharide biosynthesis gene from *Streptococcus agalactiae* Type Ia (2)

The 3.5 kb fragment of *Streptococcus agalactiae* Type Ia, which had been inserted into the plasmid pBA101 obtained in (1) of Example 1, was labeled using Random Primer DNA Labeling Kit (manufactured by Takara Shuzo) to prepare a probe.

Fragments obtained by digesting the chromosomal DNA of *Streptococcus agalactiae* Type Ia with a restriction enzyme *Bgl*II were ligated to pBluescript II SK(+) to prepare recombinant DNAs, and a library was prepared by transforming *E. coli* JM109 with these recombinant DNAs.

Using the above probe and library, colony hybridization was carried out so as to obtain a strain of clone showing a strong signal. A plasmid contained in this strain was named pBA103 and its structure was analyzed to find that it was a structure in which a 3.1 kb DNA derived from the chromosomal DNA of *Streptococcus agalactiae* Type Ia was inserted into pBluescript II SK(+). When the nucleotide sequence of the 3.1 kb DNA was determined according to the method known by persons having ordinary

skill in the art, genes named *cpsIaI* and *cpsIaJ* and a part of the *cpsIaH* gene were found in the DNA, and it was confirmed that the DNA contained in pBA103 was a DNA adjacent to the DNA contained in pBA101 on the chromosomal DNA and that pBA103 is also a plasmid which contains a part of the DNA of the capsular polysaccharide biosynthesis genes. As a result of homology search, it was confirmed that the *cpsIaI* gene and the *cpsIaJ* gene have high homology with the β1,3-*N*-acetylglucosaminyltransferase gene and the β1,4-galactosyltransferase gene, respectively (Fig. 1, *J. Bacteriol.*, *181*: 5176 (1999)).

(3) Isolation of DNA containing β1,3-galactosyltransferase gene from *Streptococcus agalactiae* Type Ib

The 3.1 kb fragment of *Streptococcus agalactiae* Type Ia, which had been inserted into the plasmid pBA103 obtained in (2) of Example 1, was labeled using Random Primer DNA Labeling Kit (manufactured by Takara Shuzo) to prepare a probe.

Streptococcus agalactiae Type Ib was cultured by the method described in *J. Bacteriol.*, *181*: 5176 (1999). After collecting the cells by centrifugation, chromosomal DNA of the microorganism was isolated and purified in accordance with the method described in *Current Protocols in Molecular Biology*.

Fragments obtained by digesting the chromosomal DNA of *Streptococcus agalactiae* Type Ib with a restriction enzyme *Bgl*II were introduced into pBluescript II SK(+) and transformed into *E. coli* JM109 to prepare a library.

Using the above probe and library, colony hybridization was carried out to obtain two cloned strains showing a strong signal. Plasmids contained in these strains were named pBB102 and pBB103, respectively, and their structures were analyzed to find that they were structures in which 5.5 and 1.4 kb fragments derived from the chromosomal DNA of *Streptococcus agalactiae* Type Ib were inserted into pBluescript II SK(+), respectively (Fig. 1).

When the nucleotide sequences of these two DNAs were determined according to the method known by persons having ordinary skill in the art, it was determined that these two DNAs were present by adjacent to each other on the chromosomal DNA and having the continued nucleotide sequence represented by SEQ ID NO:3. It was revealed that genes named *cpsIbE*, *cpsIbF*, *cpsIbG*, *cpsIbH*, *cpsIbI* and *cpsIbJ* are present in the DNA having the nucleotide sequence represented by SEQ ID NO:3. As a result of homology search, the *cpsIbE* gene showed high homology with the *cpsIaE* gene and the glucosyltransferase gene, the *cpsIbG* gene with the *cpsIaG* gene and the β1,4-galactosyltransferase gene, and the *cpsIbI* gene with the

cpsIaI gene and the β1,3-*N*-acetylgalactosaminyltransferase gene. Accordingly, it was confirmed that the DNA having the nucleotide sequence represented by SEQ ID NO:3 contains a part of the capsular polysaccharide biosynthesis genes in *Streptococcus agalactiae* Type Ib.

Also, it was considered that the *cpsIbJ* gene is a gene comprising a DNA encoding a protein having a β1,3-galactosyltransferase activity because the protein encoded by the *cpsIbJ* gene having the nucleotide sequence represented by SEQ ID NO:2 has a preserved sequence of a glycosyltransferase but its homology with the *cpsIaJ* gene is low, a *cpsIaJ* gene product takes a role in transferring galactose in the capsular polysaccharide biosynthesis in *Streptococcus agalactiae* Type Ia (*J. Bacteriol.*, *181*: 5176 (1999)), and the linkages of galactose of capsular polysaccharide in *Streptococcus agalactiae* Type Ia and *Streptococcus agalactiae* Type Ib are different, which are β1,4 and β1,3, respectively (*J. Bacteriol.*, *181*: 5176 (1999)). The amino acid sequence of the protein encoded by this DNA is shown in SEQ ID NO:1.

Example 2

Construction of a strain expressing β1,3-galactosyltransferase gene:

A DNA containing the galactosyltransferase gene obtained in (3) of Example 1 was amplified by the following

method using DNAs having the nucleotide sequences represented by SEQ ID NOs:6 and 7 synthesized using DNA Synthesizer Model 8905 manufactured by Perceptive Biosystems.

PCR was carried out using these synthesized DNAs as a primer set and the *Streptococcus agalactiae* Type Ib chromosomal DNA as the template. Using 40 μl of a reaction solution containing 0.1 μg of the chromosomal DNA, 0.5 μmol/l each primer, 2.5 units of TaKaRa LA Taq polymerase (manufactured by Takara Shuzo), 4 μl of a buffer solution for TaKaRa LA Taq polymerase and 200 μmol/l each deoxyNTP, PCR was carried out by repeating a step of 94°C for 1 minute, 42°C for 2 minutes and 72°C for 3 minutes 30 times.

After confirming amplification of the fragment of interest by subjecting 1/10 volume of the reaction solution to agarose gel electrophoresis, the amplified fragment was recovered from the remaining reaction solution using GeneClean II Kit (manufactured by Bio 101) and then dissolved in TE buffer (10 mmol/l Tris-HCl and 1 mmol/l EDTA (pH 8.0)) to obtain 20 μl of the DNA solution.

Using 5 μl of the dissolved solution, the DNA was digested with restriction enzymes *Not*I and *Xho*I, the resulting DNAs were separated using agarose gel electrophoresis and then a DNA of 2.0 kb was recovered using GeneClean II Kit.

After 0.2 μg of pBluescript II SK(+) DNA was digested with restriction enzymes *Not*I and *Xho*I, the resulting DNAs were separated by agarose gel electrophoresis and then a DNA of 3.0 kb was recovered using GeneClean II Kit.

Using a ligation kit, the 2.0 kb and 3.0 kb fragments were ligated at 16°C for 16 hours.

Using the ligation reaction solution, *E. coli* JM109 was transformed in accordance with the method known by persons having ordinary skill in the art, and the transformants were spread on LB agar medium (10 g/l Bacto Tryptone (manufactured by Difco), 10 g/l Yeast Extract (manufactured by Difco), 5 g/l sodium chloride, 15 g/l agar) containing 50 μg/ml ampicillin, followed by culturing overnight at 37°C.

By extracting plasmid from the thus grown transformant colonies in accordance with the method known by persons having ordinary skill in the art, an expression plasmid pBBPIJ was obtained. The structure of this plasmid was confirmed by restriction enzyme digestion (Fig. 2).

In the same manner, PCR was carried out using DNAs having the nucleotide sequences represented by SEQ ID NOs:7 and 8 synthesized using DNA Synthesizer Model 8905 manufactured by Perceptive Biosystems as a primer set and the *Streptococcus agalactiae* type Ib chromosomal DNA as the template.

After confirming amplification of the fragment of interest by subjecting 1/10 volume of the reaction solution to agarose gel electrophoresis, the amplified fragment was recovered from the remaining reaction solution using GeneClean II Kit (manufactured by Bio 101) to obtain 20 μl TE solution of the DNA.

Using 5 μl of the solution, the DNA was digested with restriction enzymes *Eco*RI and *Xho*I, the resulting DNAs were separated by agarose gel electrophoresis and then a DNA of 1.0 kb was recovered using GeneClean II Kit.

After 0.2 μg of pBluescript II SK(+) DNA was digested with restriction enzymes *Eco*RI and *Xho*I, the resulting DNAs were separated by agarose gel electrophoresis and then a DNA of 3.0 kb was recovered using GeneClean II Kit.

Using a ligation kit, the 1.0 kb and 3.0 kb fragments were ligated at 16°C for 16 hours.

Using the ligation reaction solution, *E. coli* JM109 was transformed in accordance with the method known by persons having ordinary skill in the art, and the transformants were spread on LB agar medium containing 50 μg/ml ampicillin, followed by culturing overnight at 37°C.

By extracting plasmid from the thus grown transformant colonies in accordance with the method known by persons having ordinary skill in the art, an expression

plasmid pBBPJ was obtained. The structure of this plasmid was confirmed by restriction enzyme digestion (Fig. 2).

Escherichia coli JM109/pBBPJ having the plasmid pBBPJ containing a DNA encoding a protein having a β1,3-galactosyltransferase activity derived from *Streptococcus agalactiae* Type Ib has been deposited as FERM BP-7400 on December 21, 2000 in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) (present name and address: International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-chome Tsukuba-shi, Ibaraki-ken, 305-8566 Japan)).

Example 3

Production of lacto-*N*-tetraose:

Each of the *Escherichia coli* JM109/pBBPIJ and JM109/pBBPJ obtained in Example 2 was inoculated into a test tube charged with 8 ml of LB medium containing 50 μg/ml ampicillin respectively, followed by culturing at 37°C for 17 hours. The culture was inoculated at 1% into a test tube charged with 8 ml of LB medium containing 50 μg/ml ampicillin respectively, followed by culturing at 37°C for 5 hours, and then IPTG was added thereto to give a concentration of 1 mmol/l. Two hours after additional

culturing, wet cells were obtained by centrifugation. A membrane fraction was prepared from the wet cells in accordance with the method known by persons having ordinary skill in the art (*J. Biol. Chem.*, *272*: 19502 (1997), *Mol. Microbiol.*, *26*: 197 (1997)). Since this membrane fraction can be stored at -80°C, if necessary, it was able to use it by thawing prior to use.

Lacto-*N*-triose II to be used as the acceptor carbohydrate was prepared by allowing lacto-*N*-neotetraose (manufactured by Sigma) to react with β-galactosidase (manufactured by Seikagaku Corporation), completely removing the non-reducing terminal galactose and then inactivating the β-galactosidase activity by heat treatment at 100°C for 5 minutes.

The reaction was carried out at 37°C for 72 hours in 0.1 ml of a reaction solution containing the JM109/pBBPIJ membrane fraction (200 μg/ml), 50 mmol/l citrate buffer (pH 7.0), 5 mmol/l $MgCl_2$, 10 mmol/l lacto-*N*-triose II and 5 mmol/l UDP-galactose.

After completion of the reaction, the reaction product was analyzed using a High-performance anion-exchange chromatography with pulsed amperometric detection system manufactured by Dionex (DX-500) under the following analyzing conditions to confirm that 0.2 mmol/l lacto-*N*-tetraose was produced and accumulated in the reaction solution.

In the same manner, it was confirmed that 0.05 mmol/l lacto-*N*-tetraose was produced and accumulated when a JM109/pBBPJ membrane fraction was used.

Analyzing conditions:

Column:	CarboPAC PA10
Eluent:	A; H_2O, B; 500 mmol/l NaOH
Gradient:	10 to 70% B in 20 min
Detector:	Pulsed amperometric detector

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. All references cited herein are incorporated, by reference, in their entirety.